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Major involvement of connexin 43 in seminiferous epithelial junction dynamics and male fertility

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ABSTRACT

In different epithelia, cell membranes contacting one another form intercellular junctional complexes including tight, adherens and gap junctions, which could mutually influence the expression of each other. We have here investigated the role of Cx43 in the control of adherens and tight junction proteins (N-cadherin, β-catenin, occludin and ZO-1) by using conditional Sertoli cell knockout Cx43 (SCCx43KO$^{-/-}$) transgenic mice and specific anti-Cx43 siRNA. Gap junction coupling and Cx43 levels were reduced in SCCx43KO$^{-/-}$ as compared to Wild-type testes. Ultrastructural analysis revealed disappearance of gap junctions, the presence of tight and adherens junctions and persistent integrity of the blood–testis barrier in SCCx43KO$^{-/-}$ testis. Occludin, N-cadherin and β-catenin levels were enhanced in SCCx43KO$^{-/-}$ mice as compared to Wild-type animals whereas ZO-1 levels were reduced. Cx43 siRNA blocked gap junction functionality in Sertoli cells and altered tight and adherens protein levels. The Cx43 control of tight and adherens junctions appeared channel-dependent since gap junction blockers (glycyrrhetinic acid and oleamide) led to similar results. These data suggest that the control of spermatogenesis by Cx43 may be mediated through Sertoli cell Cx43 channels, which are required, not only in cell/cell communication between Sertoli and germ cells, but also in the regulation of other junctional proteins essential for the blood–testis barrier.

Introduction

The molecular mechanisms by which gap junctions and their constitutive proteins, connexins (Cx), control cell proliferation and differentiation are not clearly defined. Generally gap junction channels may promote cell survival either by regulating the flux of necrotic and/or apoptotic signal effectors or by propagating diffusion of vital signals thereby contributing to cell survival (Krysko et al., 2005). It is likely that the physiological significance of Cx on cell proliferation and differentiation is the result of fine-tuned balance involving multiple elements such as the nature of Cx involved, their association to form functional channels, the communicating cell types and the hormonal control. Recent findings have also shown that hemi-channels could be involved in numerous cellular processes such as the release of ATP, NAD^+, prostaglandins and glutamate, intercellular Ca^{2+} wave propagation, cell-volume control and the passage of survival signals (reviewed in Scemes et al., 2009). Other studies tend to demonstrate that Cx, and mostly Cx43, are also able to control cell proliferation independently of their ability to form gap junction at the plasma membrane (Huang et al., 1998; recently reviewed in Cronier et al., 2009).

In the testis, proliferation, differentiation and survival of germ cells are partly dependent upon the presence of the supporting Sertoli cells that establish direct communication through Cx43, the predominant testicular Cx (Pointis and Segretain, 2005). Recently some of the present authors and others demonstrated that Sertoli cell specific Cx43 knock-out transgenic mice exhibited impaired spermatogenesis. (Brehm et al., 2007; Sridharan et al., 2007a, b). However, the molecular mechanism by which Cx43 controls spermatogenesis was not clearly identified. Previous data reported that germ cell deficiency could be associated with abnormal p53 activation (Francis and Lo, 2006) and/or mediated via the caspase-3 and the NF-kappaB pathway (Lee et al., 2006). By using an in vitro model of neonatal germ cell proliferation, we recently demonstrated that Cx43 is involved in germ cell growth by controlling spermatogonia survival rather than proliferation (Gilleron et al., 2009a). Altogether these data strongly

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support the possibility that testicular Cx43 maintains the number of germ cells by regulating Cx43 gap junction channels and communication between germ cells and the adjacent supporting cells.

Another possibility is that Cx and/or gap junctions may control the germ cell proliferation process, via indirect regulation of tight and adherens junctions, structural components of the blood–testis barrier. Indeed, there is evidence that Cx and proteins involved in cell attachment or forming tight junctions, which are highly intermingled with each other to form intercellular junctional complexes in several epithelia, could mutually influence expression and function of one another (reviewed in Derangeon et al., 2009). For example, Cx32 and Cx26 have been reported to control tight junction formation and function in several cell types, such as hepatocytes and in airway epithelial Calu-3 cells (Kojima et al., 2007). At the blood–testis barrier level, the possibility that gap junctions exert such a control on the other components of the barrier has been postulated (Lee et al., 2007; Yan et al., 2008). This hypothesis is in agreement with the previous morphological studies in continual (guinea pig) and seasonal breeder (mink) testes, which suggest that Cx43 between Sertoli cells participates in the formation and regulation of the blood–testis barrier (Pelletier, 1995). Recently, it has been reported that the blockage of Cx, by using a pan-connexin peptide that recognizes all the characterized Cx within the testis, concomitantly leads to diminished occludin and up-regulated N-cadherin expression (Lee et al., 2006). Since Cx and occludin are present at the membrane within a protein complex with ZO-1, a common adaptor shared by occludin- and connexin-associated protein complexes, it has been postulated that the dysregulation of ZO-1 could indirectly hamper the stability and integrity of the occludin associated protein complex (Lee and Cheng, 2004).

In order to provide new information on a possible relationship between the lack of Cx43, alteration of the blood–testis barrier, and germ cell deficiency, we analyzed junctional tight and adherens protein expression in Sertoli cell conditional Cx43 knock-out mice and after Cx43 gene invalidation in a Sertoli cell line by siRNA. Our data show that Cx43 gap junctions participate in the local regulation of structural proteins of the blood–testis barrier indicating a new role of Cx43 in the control of the others components of the blood barrier. The present investigation also suggests that Sertoli cell Cx43 is most probably involved in the control of the blood–testis barrier dynamics rather than in the maintenance of barrier integrity.

Materials and methods

Animals

We have produced a conditional Cx43 knockout (KO) mouse using the Cre/loxP recombination system, which lacks the Cx43 gene solely in Sertoli cells (SCCx43KO$^{-/-}$) (Brehm et al., 2007). Mice were housed with 12L/12D cycles at 25 °C and given water and food ad libitum. All animal experiments were conducted according to the standard ethical guidelines approved by the animal care committee of the Institut National de la Santé et de la Recherche Médicale (INSERM). Wild-type and SCCx43KO$^{-/-}$ transgenic mice were killed by CO_2 asphyxiation and testes were collected. Pieces of testes were directly used for gap junction coupling analysis. The remaining tissues were fixed in Bouin's solution or by immersion in methanol at −20 ° C for morphological studies or directly frozen for further Western blot and immunofluorescence analysis as described previously (Decrouy et al., 2004).

Cell culture, treatment and transfection

The SerW3 Sertoli cell line was cultured as previously described (Fiorini et al., 2008). When indicated, the gap junction blockers: 18-alpha-glycyrrhetinic acid (75 μM) and oleamide (50 μM) were added to the culture medium as we recently described (Gilleron et al., 2006), 90 min before performing gap junction functionality, immunofluorescence and Western blot of N-cadherin, β-catenin, occludin and ZO-1 analyses. To further study the effect of Cx43 depletion, SerW3 cells were transfected with a validated non-targeting siRNA or with a specific siRNA for Cx43 suppression. All anti-Cx43 siRNA probes and a negative non-silencing control siRNA conjugated with fluorescein (cat no. 1027282) were purchased from Qiagen. To design siRNA, the mRNA sequence (NCBI accession no. NM-012567) of gap junction protein, alpha 1 from rat was used. siRNAs were designed as four different siRNA duplex sequences: Rn-Gja1-1 (cat no. SI00250600), Rn-Gja1-5 (cat no. SI03071726), Rn-Gja1-6 (cat no. SI03102750), Rn-Gja1-7 (cat no. SI03118423) targeting the sequences 5′-AACAGTGCACATGTAACTAAT-3′, 5′-CAGGTAAGCTTCCCTGGTCTA-3′, 5′-GAGAGTGTTCTTTATCCAATA-3′, and 5′-TGCCGCAATTACAACAAG-CAA-3′. Control non-silencing siRNAs and anti-Cx43 siRNAs were prepared according to Qiagen technical protocol in an RNAse free environment. Cells were transfected with a transfection mixture composed of OptiMEM (Invitrogen SARL, Cergy Pontoise), Genejuice (Novagen) and 3 μg siRNA anti-Cx43/ml according to the manufacturer's instructions. Then the cells were incubated for 24–72 h post-transfection at 32 °C under 5% CO_2. The efficiency of transfection was supervised with control siRNA conjugated with fluorescein. The efficiency of suppression of Cx43 protein synthesis was determined by Western blot and immunofluorescence analyses 24 and 48 h after transfection. Among four duplexes tested, only two were efficient enough to suppress Cx43 protein synthesis (Rn-Gja1-5 and Rn-Gja1-7). For these two siRNA, no modification of the cell shape arguing for an off-target effect was observed.

Immunoblots

Testes were solubilized in NP40/Brijd 96 lysis buffer (50 mM Tris–HCl pH 7.5, 1% NP40, 1% Brij 96, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM EDTA, 1 mM Aprotinin, 25 mM Leupeptin, 1 mM Pepstatin, 2 mM Phenylmethylsulfonyl fluoride). Proteins were subjected to one-dimensional SDS-PAGE (7% and 12% of acrylamid), electroblotted onto a nitrocellulose membrane (Amersham Hybond-ECL of GE Healthcare) and analyzed by Western blotting with mouse anti-Cx43 (1:500) from Transduction Laboratories (Lexington, KY), mouse anti-Cx32 (1:1000) and mouse anti-tubulin (1:5000) from Sigma, rabbit anti-ZO-1 (1:1000), mouse anti-β-catenin (1:1000), mouse anti-N-cadherin (1:2000), mouse anti-occludin (1:2000) antibodies from Zymed Laboratories (San Francisco, CA) as previously reported (Defamie et al., 2001; Fiorini et al., 2004).The presence of the primary antibody was revealed with horseradish peroxidase-conjugated secondary antibody: anti-mouse or anti-rabbit IgG (1:10,000, Dako, Trappes, France), and visualized with an enhanced chemiluminescence detection system (ECL, Amersham, UK).

Dye coupling procedures

Gap FRAP analysis was performed as described previously (Decrouy et al., 2004; Gilleron et al., 2009b). Briefly, freshly isolated small seminiferous tubule fragments or cultured cells were incubated with 5 μM of calcein-AM (Invitrogen SARL, Cergy Pontoise, France) in DMEM for 30 min at 32 °C. Individual cells were then bleached by strong laser pulses (488 nm 100% and 50 iterations) with a Zeiss confocal microscope LSM 510 (Service Commun de Microscopie, IFR Biomédicale des Saint-Pères, Paris, France). Confocal images were taken every 3 min during a 12 min period after calcein photobleaching. Fluorescence recovery was analyzed using LSM software before bleaching, immediately afterwards, and 12 min after photobleaching. The percentage of fluorescence recovery in bleached cells was determined by averaging all cells ($n>50$) for each experiment. Similar

analyses were performed in four independent sets of experiments. We verified that the dye transfer was gap junction mediated by pre-incubating small seminiferous tubule fragments before dye coupling analysis with the gap junction coupling inhibitor alpha-glycyrrhetinic acid (75 μM).

Immunohistochemistry

Briefly, testis sections from Wild-type and SCCx43KO$^{-/-}$ mice were microwave-treated at 1000 W in sodium citrate buffer (pH 6.0) for 15 to 20 min. Sections were then blocked with 3% H_2O_2 in methanol for 30 min at room temperature (RT), with 3% skimmed milk powder (Heirler Cenouis, Radolfzell, Germany) in Tris–HCl buffer for 30 min, and finally with 5% bovine serum albumin (BSA) in Tris–HCl buffer for 30 min at room temperature. They were incubated overnight with a rabbit polyclonal anti-ZO-1 (1:500; Zymed) primary antibody at 4 °C. Sections were then exposed for 45 min to biotinylated secondary antibody, goat anti-rabbit IgG for ZO-1 (1:100; DAKO). Then sections were treated with a Vectastain Elite ABC Kit (Vector, Burlingame, CA) for 45 min. Immunoreaction was visualized using DAB solution (Biologo, Kronshagen, Germany). Sections were washed with 0.1 M Tris–HCl buffer, pH 7.4. Slices were counterstained with hematoxylin and rinsed with running water. Finally, sections were mounted with Kaiser's glycerol gelatin (Merck, Darmstadt, Germany). Control sections were treated with normal appropriate animal sera or phosphate-buffered saline (PBS), omitting the primary antibody, and were negative throughout.

Immunofluorescence analyses on tissue and cells were performed as described previously (Gilleron et al., 2009b). Briefly, seminiferous tubule fragments or SerW3 Sertoli cells were fixed in methanol at −20 °C for 6 min and permeabilized for 45 min with saponin. Then they were incubated for 2 h with mouse anti-Cx43 (1:100), mouse anti-Cx32 (1/500), mouse anti-occludin (1:100), rabbit anti-ZO-1 (1:200), mouse anti-N-cadherin (1:100) and mouse anti-β-catenin (1:100) antibodies and subsequently incubated for 1 h with goat anti-rabbit/anti-mouse FITC conjugated and goat anti-rabbit/anti-mouse TRITC conjugated antibodies (1:100). Finally, the seminiferous tubule fragments or cells were mounted in Vectashield medium with DAPI (BioValley) to label nuclei. Three-dimensional high resolution deconvolution microscopy analysis was performed with a widefield deconvolution microscope Nikon TE-2000E (SCM, University Paris Descartes) through a cooled charge-coupled device camera (Roper CoolSnap HQ2) as described previously (Segretain et al., 2003). Images were collected with NIS Element software (Nikon) and deconvoluted with AutoQuant image package algorithms. Semi-quantitative evaluation of the immunoreactive signals was performed with a specific, minicomputerized densitometric program developed for use with Visilog 4.15 image analysis software (Neosis, les Ulis, France) as described previously (Batias et al., 1999).

Electron microscopy

Testis pieces were fixed with 2.5% glutaraldehyde in phosphate-buffer saline (0.1 M, pH: 7.4) for 1 h at room temperature, postfixed in reduced osmium, dehydrated and embedded in Epon as previously reported (Lablack et al., 1998). Thin sections were counterstained with uranyl acetate and lead citrate and analyzed with a Philips CM 10 electron microscope (CNRS-UNIC, Institute A. Fessard, Gif sur Yvette, France). Evaluation of the number of intercellular junctions, identified on ultrastructural criteria, was performed as previously described (White et al., 1982).

For functional investigation of the blood–testis barrier, adult SCCx43KO$^{-/-}$, as well as adult Wild-type mice, were anesthetized with a sub-lethal intra peritoneal dose of a ketamine hydrochloride and xylazine cocktail (Brehm et al., 2007). The animals were then perfused via the left ventricle with a fixation solution containing 2% glutaraldehyde and 2% formaldehyde in 0.1 mol/l sodium cacodylate buffer adjusted to pH 7.3 according to Bergmann (1987). In order to demonstrate the existence of a functional blood–testis barrier, the fixation solution contained in addition either 10% glucose or 2% lanthanum nitrate, an electron-dense marker. Testicular tissue blocks (1 mm^3) were then immersed in the fixative without additives for 1 h, rinsed in buffer, and postfixed in an aqueous solution of 1% osmium tetroxide for 3 h. After dehydration specimens were embedded in Epon 812. Semithin sections were stained with toluidine blue, mounted in Epon 812, dried and photographed. Thin sections were contrasted with uranyl acetate and lead citrate and examined in a Zeiss EM 109 (Zeiss, Oberkochen, Germany).

Statistical analysis

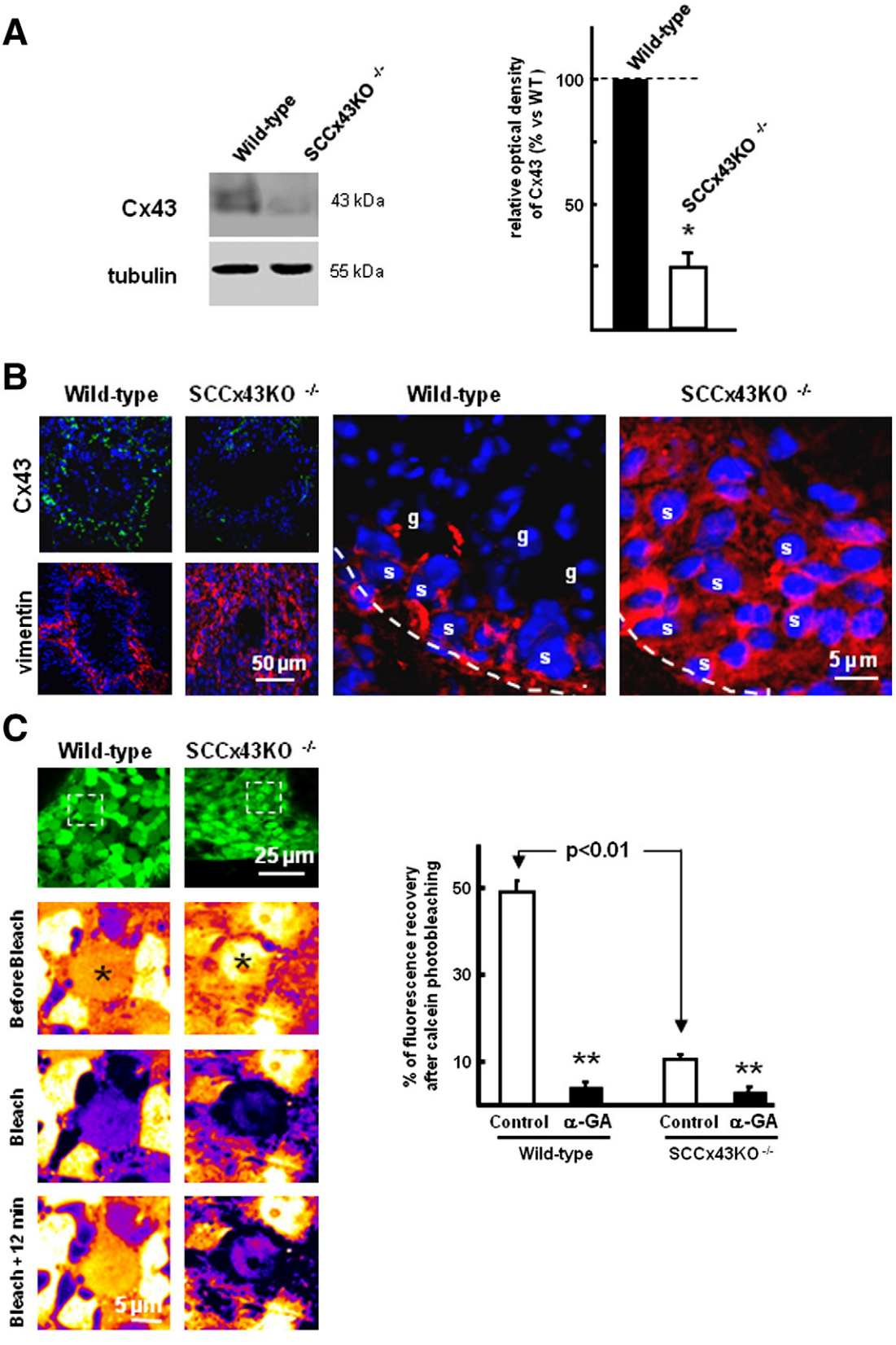
For Western blot analysis each band was quantified by densitometry with the ImageJ program and expressed as arbitrary units. Histograms show pooled data from three independent experiments. Values were expressed as the mean ± standard error of the mean (s.e.m.). Statistical analyses were assessed using one-way analysis of variance (ANOVA) and unpaired Student's *t*-test. Semi-quantitative evaluation of the immunoreactive Cx43 was expressed as percentage of the immunostaining in seminiferous tubules of Wild-type mice and presented as means ± s.e.m. of 10 seminiferous tubules analyzed in three testis sections from four different SCCx43KO$^{-/-}$ mice. For cell culture, results are the means of ± s.e.m. of four separate observations in each of three different cultures. For gap FRAP analysis at least 50 cells were analyzed for each experimental condition in four separate experiments. A one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post hoc test was used to test for differences among groups. Values were considered statistically different when $P<0.05$.

Results

We first analyzed the level of Cx43 by Western blot in the SCCx43KO$^{-/-}$ testes compared to Wild-type (Fig. 1A). As expected testicular Cx43 levels were strongly reduced in the SCCx43KO$^{-/-}$ mice as imaged by a representative Western blotting (Fig. 1A). Quantitative analysis revealed that the intensity of the immunoreactive band was significantly reduced by more than 75 ± 4% in the SCCx43KO$^{-/-}$ as compared to Wild-type testes ($P<0.05$). We then performed immunofluorescence studies to localize Cx43 (Fig. 1B). In the Wild-type testis, seminiferous tubules exhibited a complete spermatogenesis. Vimentin-positive Sertoli cells were normally present at the base of the tubules and a Cx43 immunosignal was

Fig. 1. Gap junction functionality in the testes of Wild-type and SCCx43KO$^{-/-}$ mice. (A) Testis lysates from Wild-type and SCCx43KO$^{-/-}$ mice were analyzed by Western blotting with anti-Cx43 and -tubulin antibodies. Cx43 is detected at the predicted size of 43 kDa. A representative blot is shown. Relative optical densities of the bands in arbitrary units are presented in the histogram. Results are the average of three separate experiments each done on two different mice. *$P<0.05$ significantly different from Wild-type testis. (B) Immunodetection of Cx43 (green fluorescence) and identification of Sertoli cells by vimentin analysis (red fluorescence) in the seminiferous tubules of Wild-type and SCCx43KO$^{-/-}$ mice are presented in the right panels. High magnification reveals the lack of germ cells in the transgenic animals and the invasion of seminiferous tubules by vimentin-positive Sertoli cells. Cell nuclei are identified by Dapi staining (blue fluorescence). (C) Functionality of gap junction evaluated by gap FRAP method in seminiferous tubule fragments of Wild-type and SCCx43KO$^{-/-}$ testes. Note that calcein fluorescence is totally recovered in Wild-type testes (left panels) but not in the transgenic animals (right panels). Representative of four separate experiments. Histograms represent quantitative analysis of the coupling. Note that the gap junction inhibitor, α-GA, reduced the coupling in both Wild-type and SCCx43KO$^{-/-}$ testes. **$P<0.01$ significantly different from respective controls.

detected at this level. In contrast, in conditional Sertoli cell Cx43 knock out (SCCx43KO$^{-/-}$) mice, verified by the absence of immunoreactive signal for Cx43, seminiferous tubules were empty of germ cells and numerous vimentin-positive cells were observed (Fig. 1B, left panels). High magnification clearly shows the presence of large clusters of vimentin-positive and Cx43-negative cells with shaped nuclei characteristic of Sertoli cells (Fig. 1B, right panels), which invaded the seminiferous epithelium. In contrast to Wild-type seminiferous



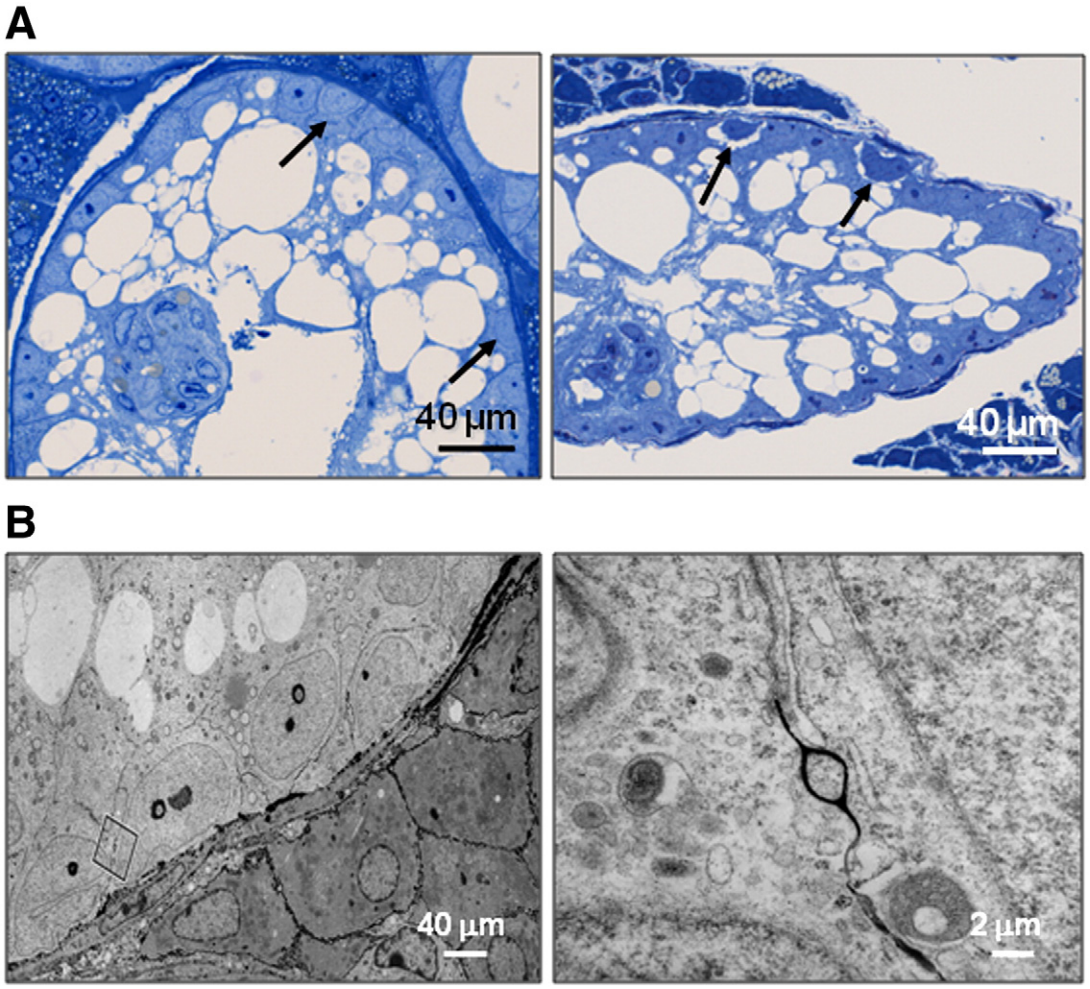


Fig. 2. Functionality analysis of the blood–testis barrier in the testes of adult SCCx43KO$^{-/-}$ mice. (A) Perfusion of adult SCCx43KO$^{-/-}$ mice with a fixation solution containing glutaraldehyde and formaldehyde resulted in no shrinkage artefacts in spermatogonia (arrows, left panel). However, perfusion of SCCx43KO$^{-/-}$ mice with a hypertonic fixation solution containing 10% glucose revealed shrinkage artefacts only in spermatogonia indicating an effective barrier (arrows, right panel). (B) In a second experiment, adult SCCx43KO$^{-/-}$ mice were perfused with a fixation solution that contains 2% lanthanum nitrate. This tracer moved within the intercellular spaces of interstitial Leydig cells and when entering the seminiferous epithelium via the basal membrane the tracer was stopped by functional tight junctions formed by Sertoli cells (left and enlargement of inset right) confirming the existence of an effective blood testis barrier in adult SCCx43KO$^{-/-}$ mice.

tubules, at least four rows of vimentin-positive cells and the total absence of vimentin-negative germ cells were reported in the SCCx43KO$^{-/-}$ seminiferous tubules.

To verify if Sertoli cells were functionally coupled, gap FRAP analyses were performed on seminiferous tubules from Wild-type and SCCx43KO$^{-/-}$ mice (Fig. 1C). Calcein fluorescence recovery was rapidly observed within 12 min after photobleaching in Wild-type testes. In contrast, the level of fluorescence recovery was markedly less in SCCx43KO$^{-/-}$ seminiferous tubules as demonstrated by the lack of fluorescence recovery and imaged in this representative experiment. Quantitative analysis performed in at least 50 cells in four separate experiments confirmed that the number of coupled cells was significantly reduced in SCCx43KO$^{-/-}$ testes as compared to Wild-type testes ($P<0.01$).

To make sure that the return of fluorescence was mediated through gap junction channels, seminiferous tubule fragments were treated with a gap junction blocking agent, α-GA (Fig. 1C, right panel). In this condition, the coupling was markedly affected in both Wild-type and transgenic mice by the gap junction blocker, supporting the presence of functional gap junctions. A remaining coupling was, however, still persistent in SCCx43KO$^{-/-}$ seminiferous tubules suggesting that Cx43 was not totally abolished in the mutant mice or that other testicular Cx could supply Cx43 function.

Among the Cx present in Sertoli cells and capable of participating in the control of germ cell, we recently demonstrated that Cx32 is also involved in cell proliferation within the seminiferous epithelium (Gilleron et al., 2009a). Thus, we further analyzed Cx32 expression. Our data revealed that the level of Cx32 protein, analyzed by Western blot studies was reduced by about 50% in testes of the SCCx43KO$^{-/-}$ transgenic mice (Supplemental Fig. 1). Immunofluorescence analyses of Cx32 confirmed these data indicating that the Cx32 immunosignal found in the basal compartment of seminiferous tubules in control mice was quite undetectable in SCCx43KO$^{-/-}$ seminiferous tubules (Supplemental Fig. 1).

As Cx43 in Sertoli cells is believed to play an essential role in the control of spermatogenesis, we hypothesized that deletion of this Cx may lead to an alteration of the blood–testis barrier function, providing a possible explanation for the impaired spermatogenesis in SCCx43KO$^{-/-}$ testes. To investigate the integrity of the blood–testis barrier, two experimental approaches, which allow us to appreciate the integrity of the barrier (response to a 10% glucose hypertonic shock or use of an electron-dense tracer), were developed (Fig. 2). As we previously reported, the seminiferous tubules of SCCx43KO$^{-/-}$ mice exhibited major signs of adluminal vacuolization and some clusters of Sertoli cells were observed within tubules (Brehm et al., 2007). Rare germ cells, recognizable by their small round nucleus with no large nucleoli inside, were also noticed at the

base of the tubule (Fig. 2A). When adult SCCx43KO$^{-/-}$ mice were perfused with a fixation solution containing glutaraldehyde and formaldehyde in the absence of glucose no shrinkage artefacts were observed (Fig. 2A, left panel). In contrast, perfusion of SCCx43KO$^{-/-}$ mice with a hypertonic fixation solution containing 10% glucose revealed that shrinkage artefacts occurred only at the contact between germ cells and Sertoli cells that lie under the barrier and that are close to the basal lamina membrane (Fig. 2A, right panel). The absence of vacuoles between adjacent Sertoli cells was indicative of an effective barrier. In the second experiment, adult SCCx43KO$^{-/-}$ mice were perfused with a fixation solution containing 2% lanthanum nitrate (Fig. 2B). The electron-dense tracer was able to move within the intercellular spaces of interstitial Leydig cells (Fig. 2B, left panel). However, tracer penetration into the adluminal compartment of the seminiferous epithelium was blocked by functional tight junctions (Fig. 2B, enlargement of inset). Together, these results confirm the existence of an effective barrier between Sertoli cells in adult SCCx43KO$^{-/-}$ mice.

To further analyze whether the lack of Cx43 in SCCx43KO$^{-/-}$ testes alters the expression of different proteins of the blood–testis barrier, ZO-1, β-catenin, N-cadherin and occludin were analyzed by Western blotting and immunofluorescence. Immunoblots of three different control and transgenic mice were presented in Fig. 3A. The intensities of the bands for ZO-1 were markedly reduced in the three testes of the conditional Sertoli cell KO mice. In contrast, for the three other junctional proteins analyzed the levels of the immunosignal were more elevated in the transgenic mice as compared to Wild-type animals (Fig. 3A, left panel). Quantitative analyses of the relative optical density measured in three separate experiments revealed that the ZO-1 level was slightly reduced ($P<0.05$) whereas β-catenin, N-cadherin and occludin levels were at least two times higher than controls ($P<0.01$) (Fig. 3A, right panel). To verify these results immunofluorescence analyses of ZO-1, β-catenin, N-cadherin and occludin were performed in testis sections of Wild-type and SCCx43KO$^{-/-}$ testes (Fig. 3B). Images at low magnification, representative of several seminiferous tubules revealed the presence of the four proteins in the testis cross-sections with apparent stronger immunosignal in the transgenic animals as compared to Wild-type mice (inset). High magnification showed that, as expected, ZO-1 was expressed in the testis of Wild-type animals and formed a continuum in the longitudinal plane (Fig. 3C). However, the reduction in ZO-1 levels observed by Western blotting was not so clearly obvious in SCCx43KO$^{-/-}$ mice. In contrast, high magnification photomicrographs revealed that the intensities of the immunolabeling for β-catenin, N-cadherin and occludin were higher in cross seminiferous tubule sections of SCCx43KO$^{-/-}$ as compared to Wild-type mice. In addition, the immunosignal for the four proteins was more diffuse and distributed between clusters of Sertoli cells in SCCx43KO$^{-/-}$ mice as compared to the confined signals mainly present at the basal compartment of the Wild-type seminiferous tubules (high magnification of the area delimited by dotted lines). Quantitative analyses indicate that the immunoreactive signals were increased by $25\pm1\%$ for N-cadherin ($P<0.01$), $20\pm3\%$ for β-catenin ($P<0.01$) and $33\pm5\%$ for occludin ($P<0.01$) (supplemental Fig. 2A).

Ultrastructural examination of SCCx43KO$^{-/-}$ seminiferous epithelium, performed to carefully analyze the fine structural organization of the barrier, showed that Sertoli cells were classically organized with elongated and indented nuclei with nucleoli, small Golgi cisternae, numerous mitochondria, and endoplasmic reticulum cisternae (Fig. 4A). However, while Wild-type Sertoli cell cytoplasms presented an elongated organization and surrounded the numerous germ cells, SCCx43KO$^{-/-}$ Sertoli cells were more flattened toward the base of the tubules and rare germ cells were located basally. At low magnification, the blood–testis barrier appeared quite similar, but was more developed in SCCx43KO$^{-/-}$ testes. Analysis of gap, tight and adherens junctions, identified by their typical ultrastructural aspects (Fig. 4B, upper panels) indicated some differences in the number of junctions present at the blood–testis barrier level between Wild-type and transgenic mice. In the SCCx43KO$^{-/-}$ testis, gap junctions were not easily detectable, but numerous tight and adherens junctions were observed as compared to Wild-type testis. Quantitative analysis of the three types of junctions per unit length of the blood–testis barrier supported the expected disappearance of gap junctions ($P<0.01$) and confirmed the increase in the number of tight ($P<0.05$) and adherens junctions ($P<0.01$) between SCCx43KO$^{-/-}$ Sertoli cells (Fig. 4B, lower right panel).

To validate the effect of Cx43 in the SCCx43KO$^{-/-}$ transgenic mice, Cx43 gene expression was invalidated by RNAi technique in the SerW3 Sertoli cell line, known to express endogenous Cx43 (Fiorini et al., 2008). Before analyzing tight and adherens protein levels the effect of Cx43-siRNA was examined on both Cx43 protein and gap junction coupling. Among the four different siRNA duplex sequences tested, two of them (Rn-Gja1-5 and Rn-Gja1-7) markedly inhibited both Cx43 protein level and coupling. A representative Western blot demonstrated that the level of Cx43 was lower in cells transfected with the two anti-Cx43 siRNA (Fig. 5A, left panel). Quantification of the signal revealed that the intensities of the Cx43 bands were markedly reduced by $65\pm3\%$ after transfection with the anti-Cx43 siRNA Rn-Gja1-7 ($P<0.01$) and by $68\pm2\%$ with the anti-Cx43 siRNA Rn-Gja1-5 ($P<0.01$). As shown in Fig. 5B calcein fluorescence recovery was rapidly obtained within 12 min after photobleaching in control cells transfected with the non-silencing siRNA whereas the level of fluorescence recovery was markedly reduced in cells transfected with the two anti-Cx43 siRNA Rn-Gja1-7. Similar results were obtained with the second siRNA (data not shown).

Representative Western blots of tight and adherens junction proteins indicated that the two anti-Cx43 siRNAs increased the levels of N-cadherin, β-catenin and occludin without any significant change of ZO-1 level (Fig. 6A, left panel). Quantitative analysis of three independent experiments pointed out a significant increase ($P<0.05$) in the levels of N-cadherin, β-catenin and occludin in cells transfected with the Rn-Gja1-7 anti-Cx43 siRNA. In contrast, ZO-1 levels were not altered (Fig. 6A, right panel). Identical data were obtained with the second siRNA tested. In agreement with these results, the intensities of the immunofluorescence signals for N-cadherin, β-catenin and occludin were higher in cells transfected with the anti-Cx43 siRNA (Rn-Gja1-5) as compared to control cells transfected with the non-silencing siRNA (Fig. 6B). Quantitative analyses indicate that the immunoreactive signals were increased by $30\pm4\%$ for N-cadherin ($P<0.01$), $11\pm6\%$ for β-catenin ($P<0.01$) and $23\pm4\%$ for occludin ($P<0.01$) (supplemental Fig. 2B). Identical effects were obtained with the anti-Cx43 siRNA Rn-Gja1-7 (data not shown).

In order to determine if the effect of Cx43 could be dependent or independent of Cx43 gap junction channel integrity, Sertoli cells were subjected to oleamide and α-GA, two potent inhibitors of gap junction coupling. In our experimental conditions, the two inhibitors tested blocked the coupling between adjacent cells as compared to untreated cells (Fig. 7A, left panel). This inhibitory effect on coupling occurred rapidly and was maintained for longer time periods when the inhibitors were present in the culture medium (Fig. 7A, right panel). The levels of ZO-1, β-catenin, N-cadherin and occludin were then analyzed by Western blotting. A blot representative of two different cultures in three separate experiments is presented in Fig. 7B (left panel). The results showed that both gap junction coupling inhibitors increased the levels of β-catenin, N-cadherin and occludin, as compared to tubulin used as control. Quantitative analysis revealed that the intensity of the immunoreactive bands for β-catenin, N-cadherin and occludin was significantly enhanced ($p<0.01$) (Fig. 7B, right panel). In contrast, ZO-1 levels were unaffected by α-GA exposure or slightly reduced by oleamide treatment ($p<0.05$).

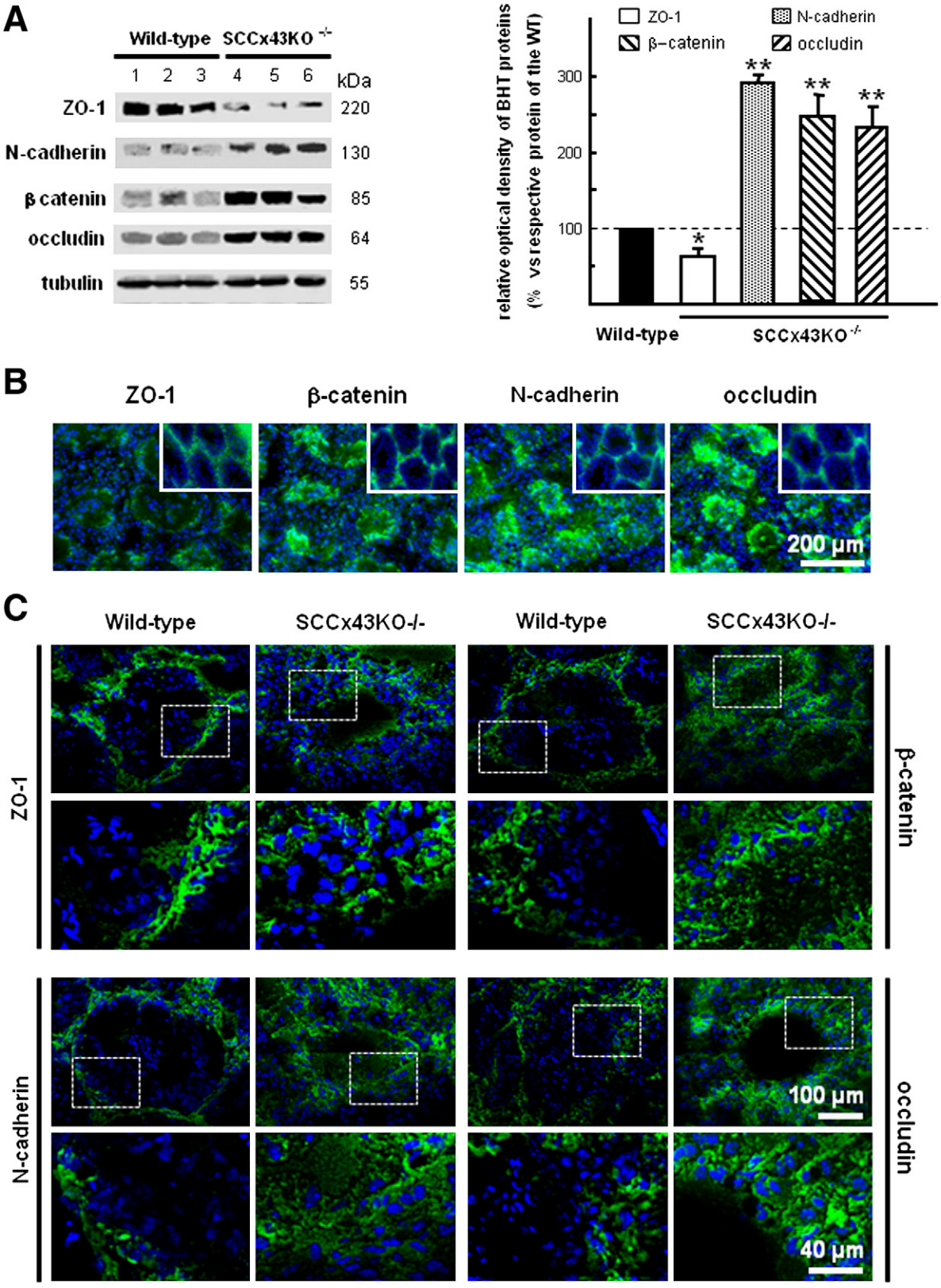


Fig. 3. Analysis of tight and adherens protein components in the testes of adult SCCx43KO$^{-/-}$ mice. (A) Testis lysates from Wild-type and SCCx43KO$^{-/-}$ mice were analyzed by Western blotting with anti-ZO1, -β-catenin, -N-cadherin, -occludin and -tubulin antibodies. All the proteins analyzed are detected at their predicted sizes of 220, 130, 85, 64, and 55 kDa respectively. The numbers indicated in the top of the blot correspond to separate testis lysates from three Wild-type or SCCx43KO$^{-/-}$ animals. A representative blot of three different experiments is shown. Relative optical densities of the bands in arbitrary units are presented in the right panels. Results are means ± s.e.m. of three different experiments. *P<0.05, **P<0.01 significantly different from the respective protein in Wild-type animals. (B) The upper panels represent ZO-1, β-catenin, N-cadherin, occludin immunosignals (green fluorescence) in testis cross-sections of SCCx43KO$^{-/-}$ or of Wild-type mice (insets). (C) High magnification of representative seminiferous tubules of Wild-type and SCCx43KO$^{-/-}$ animals. For each protein the lower panels correspond to high magnification of the area delimited by dotted lines. Cell nuclei are identified by Dapi staining (blue fluorescence). Representative of three different observations in three different animals for each case.

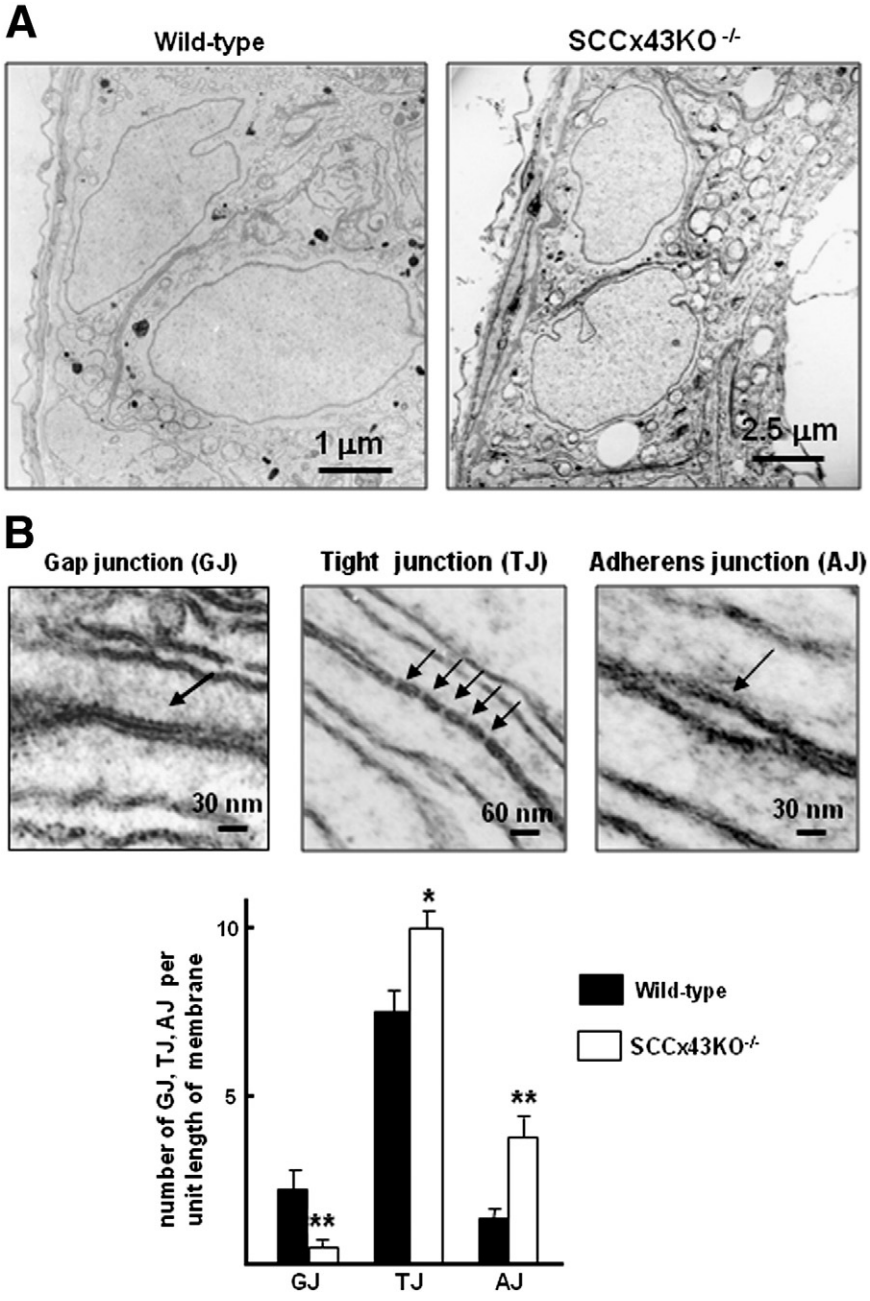


Fig. 4. Electron microscopy analysis of gap, tight and adherens junctions in Wild-type and SCCx43KO$^{-/-}$ mice. (A) Electron microscopy of Sertoli cells in Wild-type and SCCx43KO$^{-/-}$ mice. Note the absence of germ cells in the adluminal region of the transgenic seminiferous tubules and the vacuolar organization of the epithelia (left panels). (B) Large magnification allows to clearly determine the ultrastructure of the blood–testis barrier and to identify gap (left panel), tight (middle panel) and adherens (right panel) junctions. Quantitative analysis of the three different types of junctions between Wild-type and SCCx43KO$^{-/-}$ Sertoli cells per unit length of the blood–testis barrier is indicated by histograms. Results are means ± s.e.m. of three different experiments. *P<0.05, **P<0.01 significantly different from Wild-type animals.

Discussion

The blood–testis barrier is a highly controlled dynamic structure that opens and closes to allow the migration of germ cells across this barrier (Yan et al., 2008). At this level, adherens, tight and gap junctions are closely intermingled and their protein components are essential for maintenance of spermatogenesis. Thus, the dynamic processes that lead to the formation and the rupture of the testis junctions and that locally control the expression of these junctional protein components are key events of spermatogenesis. Clear evidence is now accumulating that proteins structuring gap, tight and adherens junctions can interact between themselves and thus control each other's expression and function (reviewed in Derangeon et al., 2009). A Sertoli cell specific knock-out of Cx43 has been produced using the Cre-loxP methodology for analyzing the role of Cx43 in the control of spermatogenesis (Brehm et al., 2007; Sridharan et al., 2007a, b). By using this transgenic model we have sought to examine the role of Cx43 in controlling components of tight and adherens junctions. We report herein that conditional invalidation of Cx43 only in Sertoli cells, which was associated with reduced Cx43 protein level and with a dramatic decrease in gap junction coupling, was able to significantly alter the expression of junctional proteins such as N-cadherin, β-catenin, occludin and ZO-1. Blockage of coupling with specific inhibitors of gap junction (oleamide and α-GA) or experimental decrease of Cx43 using specific siRNA in a Sertoli cell line led to similar effect. These data suggest that Cx43 based channels are required for the maintenance of junctional proteins essential for blood–testis barrier integrity and spermatogenesis.

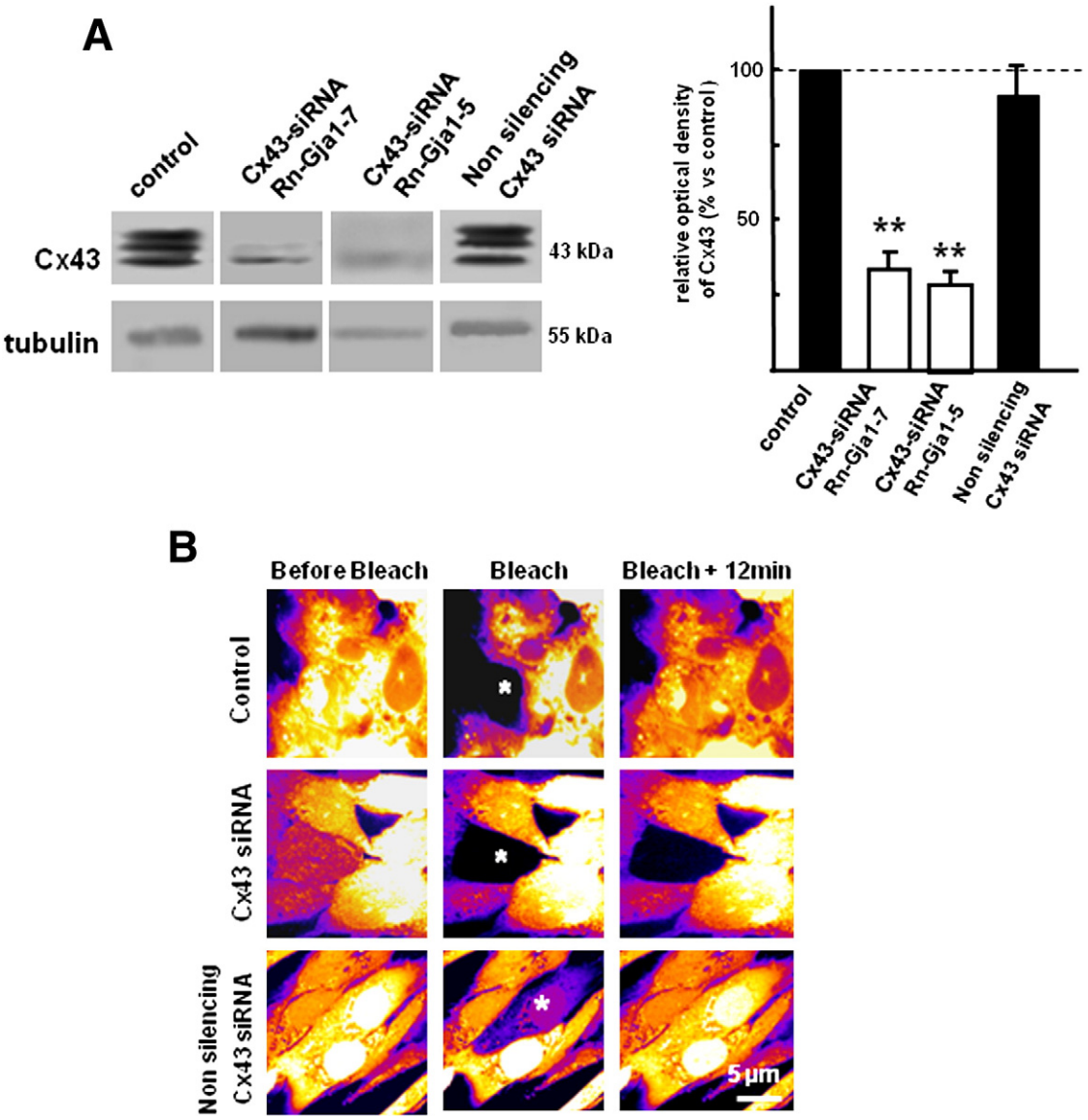


Fig. 5. Effect of anti-Cx43 siRNA on Cx43 protein levels and on gap junction functionality in cultured Sertoli cells. (A) The efficiency of suppression of Cx43 protein synthesis by two siRNA was verified by Western blot. A representative immunoblot is presented on the left panel. Relative optical densities of the bands in arbitrary units are presented in the right panels. Results are means ± s.e.m. of three different experiments. $^{*}P<0.05$, $^{**}P<0.01$ significantly different from the respective protein in cells transfected with the control non silencing siRNA. (B) A representative image of Dye coupling analysis, as described in Materials and methods, in control and SerW3 Sertoli cells transfected with anti-Cx43 siRNA Rn-Gja1-7 is presented in the right panel. Note that calcein fluorescence is noticeably recovered in photobleached control cells but not in Cx43 siRNA transfected cells.

Although the blood–testis barrier is slightly different from the other epithelial barriers, due to its opening and closing during specific stages of spermatogenesis, its structural organization shows similarities with the junctional complexes present in different types of epithelia, allowing us to propose that the control exerted by one component on the others could be also applied to other epithelial intercellular junctional complexes.

The possibility that the assembly of adherens and tight junction proteins represents a prerequisite for gap junction formation has been largely debated. Indeed, interaction between Cx43 and β-catenin is essential for the formation of gap junctions in Xenope embryos (Krufka et al., 1998). In several cell types antibodies directed against the extracellular domain of Cx43 or N-cadherin prevented the formation of adherens and gap junctions (Meyer et al., 1992; Frenzel and Johnson, 1996). The possibility that adhesion molecules influence the intracellular trafficking of Cx and function rather than expression has been suggested (Jongen et al., 1991; Hernandez-Blazquez et al., 2001; Govindarajan et al., 2002). A reciprocal regulation of the tight junction protein claudin 1 and Cx43 has been also demonstrated in human astrocytes subjected to IL-1β (Duffy et al., 2000). In turn, other lines of evidence reported that gap junctions could control the expression and/or function of adherens and tight junction molecules (reviewed in Derangeon et al., 2009). For example previous in vitro studies demonstrated that transfection of Cx32 cDNA, in a mouse hepatocyte cell line which lacked expression of Cx26 and Cx32, increased the expression of the integral tight junction proteins (occludin, claudin-1 and -2) and the number of tight junction strands (Kojima et al., 1999). In the testis, such a possibility may also occur since the blockage of Cx functions by using pan-connexin peptide, which recognizes all the testicular connexins, concomitantly leads to diminished occludin and increased N-cadherin expressions (Lee et al., 2006). These data were in agreement with previous histological observations in continual (guinea pig) and seasonal breeder (mink) testes, showing that Cx43 between Sertoli cells participates in the formation and regulation of the blood–testis barrier (Pelletier, 1995).

The present data revealed that occludin, N-cadherin and β-catenin levels were increased in the testes of SCCx43KO$^{-/-}$ mice suggesting

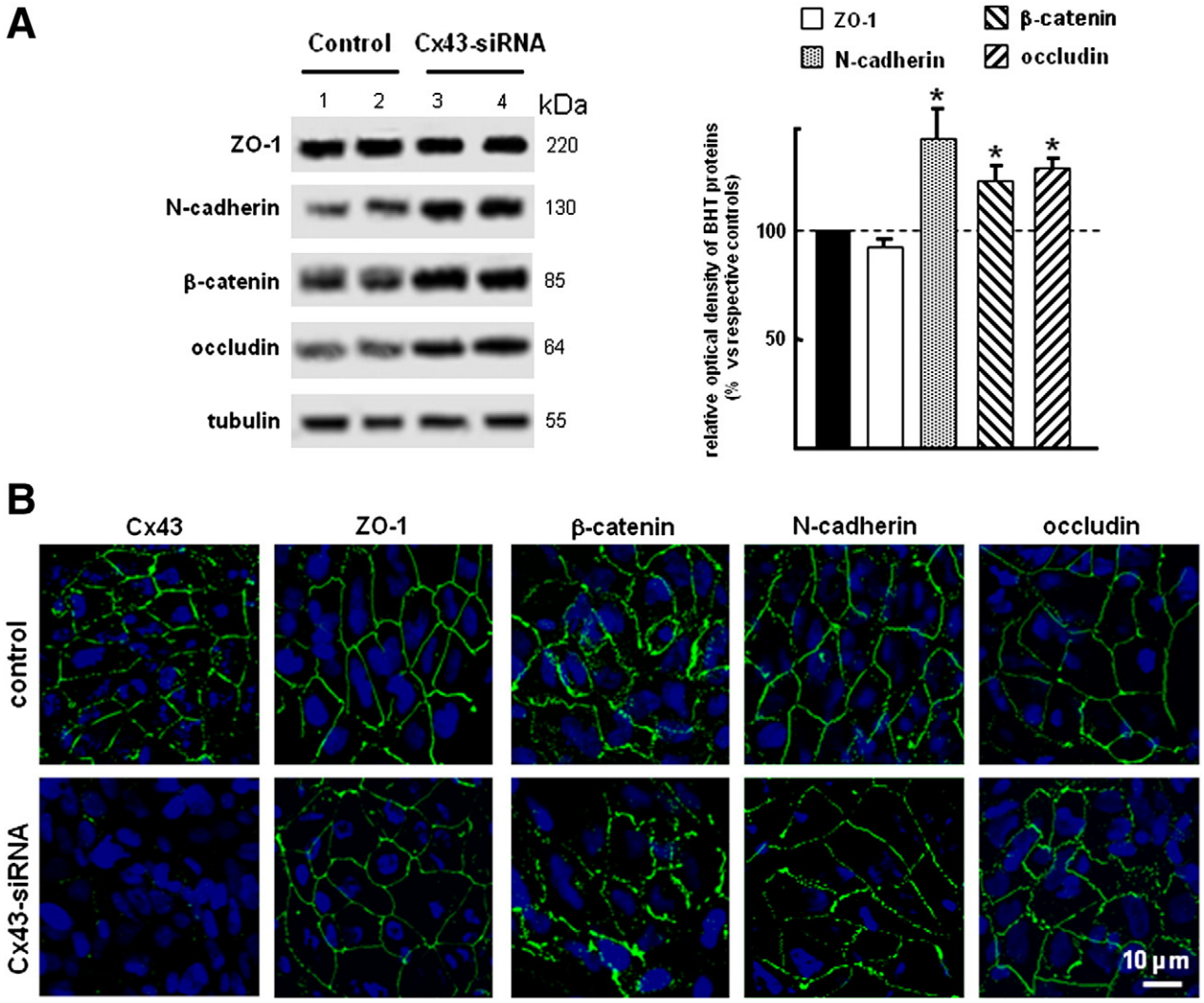


Fig. 6. Effect of anti-Cx43 siRNA on Cx43, ZO-1, β-catenin, -N-cadherin, -occludin protein levels in cultured Sertoli cells. (A) Lysates from controls (lines 1,2) or cells transfected with the two anti-Cx43 siRNA (lines 3,4) were analyzed after 48 h by Western blotting with anti-ZO1, -β-catenin, -N-cadherin, -occludin and -tubulin antibodies. All the proteins analyzed are detected at their predicted sizes of 220, 130, 85, 64, and 55 kDa respectively. A representative blot with the two different siRNA is shown. The relative optical densities of the bands in arbitrary units for Sertoli cells transfected with anti-Cx43 siRNA Rn-Gja1-7 are presented in the right panel. Results are means ± s.e.m. of three separate experiments. *$P<0.05$, significantly different from the respective control cells transfected with the non silencing siRNA. (B) Immunolocalization of Cx43, ZO-1, β-catenin, N-cadherin, occludin immunosignals in control and in Sertoli cells transfected with anti-Cx43 siRNA Rn-Gja1-7. Cell nuclei are identified by Dapi staining (blue fluorescence). Representative of three separate experiments.

that Cx43 may control and down-regulate tight and adherens junctions. These alterations of junctional proteins were associated with a reduction of ZO-1, a common adaptor of tight junction proteins (occludin, claudins and JAMs), of proteins linked to the actin cytoskeleton (β-catenin, afadin) and of Cx (Lee and Cheng, 2004). Although the mechanisms by which Cx43 controls ZO-1 are not established one can speculate that in the absence of Cx43, which links ZO-1, unbound ZO-1 levels could increase and then down-regulate its expression. The discrepancies observed between the in vivo and in vitro effects of Cx43 gene deletion on ZO-1, could be due to the relatively short-latency effect of the Cx43 siRNA in cultured Sertoli cells versus longer response time in the transgenic Cx43 KO model. It is also possible that these controversial results reflect non-physiological issues. This possibility is supported by several observations. First, such a difference between in vivo and in vitro studies was not reported for occludin, N-cadherin and β-catenin. Second, α-GA, a potent inhibitor of gap junction coupling increased in vitro the expression of these proteins without affecting ZO-1 levels. Third, Western blotting experiments were performed on fragments of whole testis, containing different cell types with different ratios, which could change between Wild-type and SCCx43KO$^{-/-}$ testis, whereas siRNA experiments concerned only one cell type. In order to confirm this possibility we looked for the cell types that express ZO-1 in the transgenic testis (supplemental Fig. 3). As expected and in agreement with Fig. 3, the ZO-1 signal was specifically located between Sertoli cell and sometimes around blood vessels, but was absent in Leydig cells. Thus, altogether these data suggest that the reduced ZO-1 levels in Western blots could be due to a dilution of the signal by proteins from Leydig cells, which populate the inter-tubular compartments more intensively than Sertoli cells proliferate in the SCCx43KO$^{-/-}$ testis (supplemental Fig. 3). This was also verified when ZO-1 levels were expressed with regard to vimentin, a specific marker of somatic cells (supplemental Fig. 4).

Our findings are not able to confirm the previous results of Lee et al. (2006) who reported that intratesticular injection of pan-connexin inhibitory peptide, which recognizes virtually all the identified Cx in the testis, reduced the expression of occludin. Such a discrepancy might be explained by the difference between the non-specificity of Cx43 invalidation in pan-Cx peptide injected testis, in which all testicular cells expressing Cx43 may be affected,

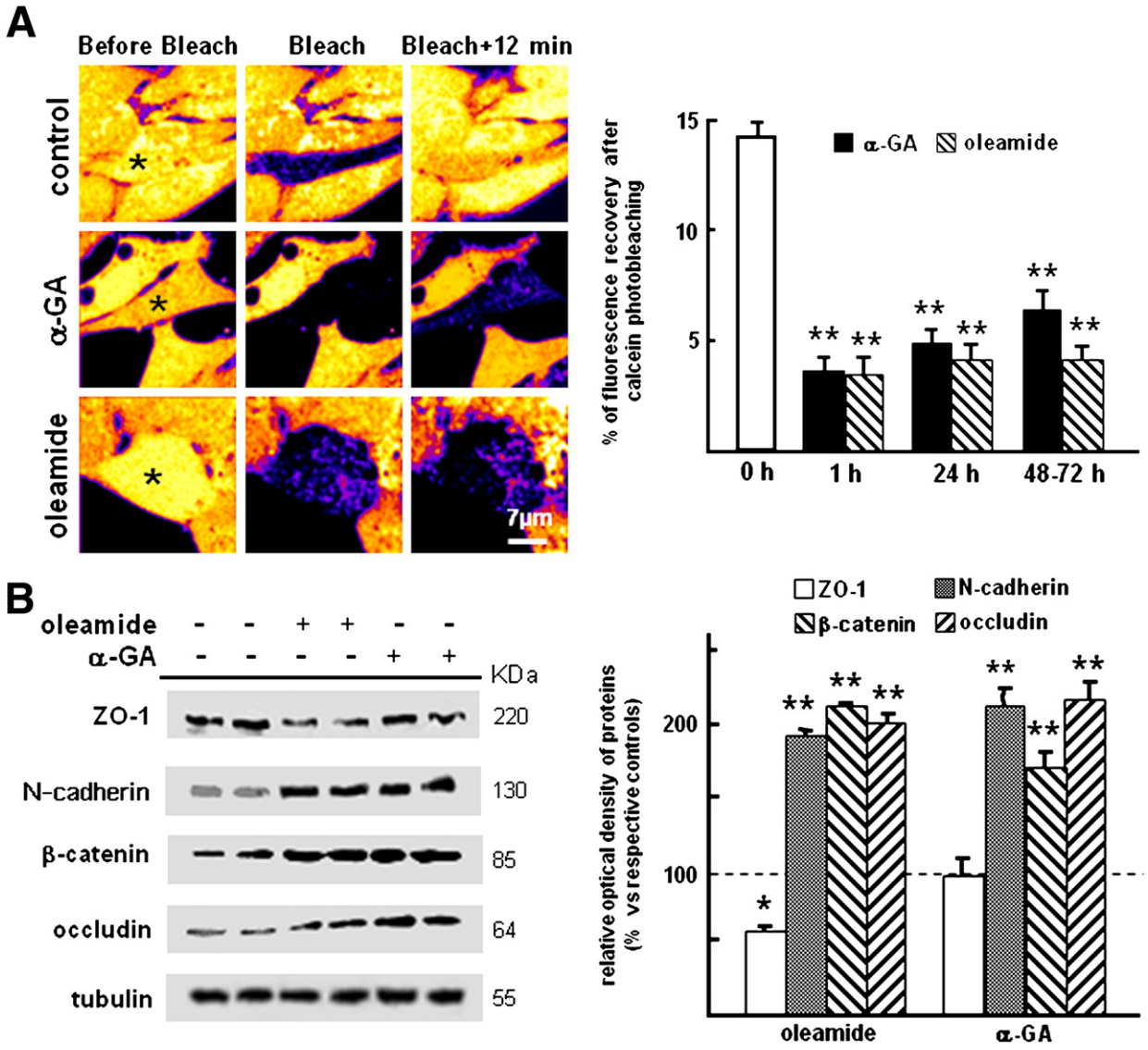


Fig. 7. Effect of two potent blockers of gap junction coupling (α-GA and oleamide) on ZO-1, β-catenin, N-cadherin, occludin in cultured Sertoli cells. (A) Dye coupling analysis, as described in Materials and methods, in control and SerW3 Sertoli cells treated with α-GA (100 µmol/l) or oleamide (50 µmol/l). Note that calcein fluorescence is noticeably recovered in photobleached control cells (asterisk) but not in α-GA or oleamide exposed cells. The histograms on the right represent dye transfer efficiency expressed as % of fluorescence recovery after calcein photobleaching in Sertoli cells cultured for 1, 24 or 48–72 h in the presence of the different blockers. Representative of four separate experiments. $^{**}P<0.01$ was significantly different from control cells. (B) Lysates from control, α-GA and oleamide exposed cells for 72 h were analyzed by Western blotting with anti-ZO1, -β-catenin, -N-cadherin, -occludin and -tubulin antibodies. All the proteins analyzed are detected at their predicted sizes of 220, 130, 85, 64, and 55 kDa respectively. A representative blot with two different duplicate for each condition is shown. The relative optical densities of the bands in arbitrary units are presented in the right panel. Results are means ± s.e.m. of three separate experiments. $^{*}P<0.05$, $^{**}P<0.01$ significantly different from control untreated cells.

compared with the current specific invalidation of Cx43 gene affecting only Sertoli cells. In addition, the brief time-period during which Cx43 is altered, shortly after injection, and the fact that only the cells near the injection site might be affected could also explain these different results, since in the SCCx43KO$^{-/-}$ mice all the Sertoli cells are affected.

The Sertoli cell specific knock-out of Cx43 using the Cre-loxP technology was performed to analyze the role of Cx43 in the control of spermatogenesis (Brehm et al., 2007; Sridharan et al., 2007a, b). This model allowed us to demonstrate that Cx43 is an essential component of spermatogenesis involved in the control of proliferation and differentiation of Sertoli cells as also verified here by the large clusters of intratubular Sertoli cells and the lack of most germ cells (spermatogonia) within the testes of SCCx43KO$^{-/-}$ mice. The low recovery of the dye fluorescence reflecting a relative coupling between adjacent cells in SCCx43KO$^{-/-}$ transgenic testis, reported in the current study, does not appear enough to control germ cell maturation since most seminiferous tubules were devoid of any germ cells. This residual coupling could be due to persistent levels of Cx43 or to the presence of other Cx described in the testis (reviewed in Pointis and Segretain, 2005). Previous reports in specific cell types revealed that the loss of function of a Cx can be compensated by other Cx (Minkoff et al., 1999) and that one Cx could regulate the expression of other Cx members (Nakase et al., 2004; Lee et al., 2006). Such compensation is unlikely in the current study since Western blot and immunofluorescence analyses revealed that the loss of Cx43 in SCCx43KO$^{-/-}$ testis was accompanied by a concomitant but lower reduction of Cx32, another Sertoli cell Cx involved in the control of spermatogenesis (Gilleron et al., 2009a). Similarly, reduced expression of Cx26 has been reported in connexin32-defective liver (Nelles et al., 1996). The present findings also agree with other data in knock-in mice demonstrating that Cx26 and Cx32 cannot functionally replace Cx43 (Plum et al., 2001; Winterhager et al., 2007). Since, none of the testicular Cx, apart from Cx43, plays a crucial role in spermatogenesis

(Pointis et al., 2009), one can speculate that Sertoli cell Cx43 must exert a unique regulatory role in this process.

The accurate mechanisms by which Cx43 exerts its effect on spermatogenesis are, however, unknown. In addition to allowing communication between somatic and germ cells and the formation of hemi-channels that could act as paracrine conduits to spread factors that modulate the fate of germ cells, there is also possibility that Cx43 and/or Cx43 based gap junctions may control the germ cell proliferation process through regulation of tight and adherens junction components could be hypothesized. Indeed, such a putative regulation hypothesis has been proposed in several cell types, for example hepatocytes (Kojima et al., 1999; 2002), immortalized mouse hepatic cells (Kojima et al., 2002) and in human airway epithelial Calu-3 cells (Go et al., 2006), in which reduced expression and delocalization of claudin 1, claudin 2 and occludin have been reported. In contrast, at the blood–brain barrier Cx43-based gap junctions might be required to maintain the endothelial barrier function without altering the expression of occludin, claudin 5 and ZO-1 (Nagasawa et al., 2006).

The present findings showed that the invalidation of Cx43 in SCCx43KO$^{-/-}$ mice or in Sertoli cells by specific siRNA, mainly altered the levels of occludin, N-cadherin and β-catenin. In the testis, spermatogonia located outside of the blood–testis barrier must differentiate into preleptotene/leptotene spermatocytes, which migrate from the basal to the adluminal compartment of the seminiferous epithelium, traversing the blood–testis barrier in late stage VIII–early stage IX (Russell, 1977; Yan et al., 2008). These dynamic processes that require establishment and rupture of the testis junctions are key events of spermatogenesis. The present observations reveal that the basal germ cells present within the SCCx43KO$^{-/-}$ seminiferous tubules are unable to pass the blood–testis barrier to continue their differentiation suggesting that Cx43 could act as a signaling molecule involved in the dynamic opening of the blood–testis barrier. This hypothesis is strongly supported by a recent study demonstrating that Cx43 associated with the desmosomal protein plakophilin-2, can participate in the control of the blood–testis barrier dynamics (Li et al., 2009a).

The increased levels of occludin, N-cadherin and β-catenin within the testis could reinforce the interaction between Sertoli cells at the level of the blood–testis barrier and impair the dynamic process of opening and closing of this barrier for normal progression of spermatogenesis. This possibility is supported by the current data on blood–testis barrier functionality, analyzed by lanthanum tracer and hypertonic glucose perfusion of the testis, demonstrating an effective barrier between Sertoli cells in the SCCx43KO$^{-/-}$ testis.

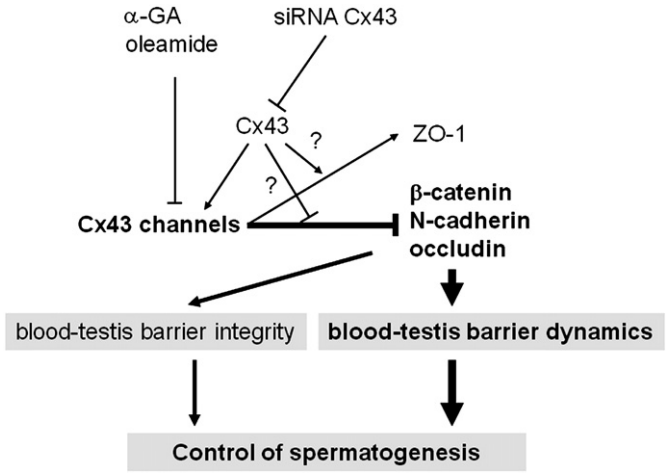


Fig. 8. Schematic drawing illustrating the mechanisms by which Cx43 can control ZO1, β-catenin, N-cadherin, and occludin proteins at the blood–testis barrier level and their consequences on spermatogenesis.

There is also the possibility that a permanent blood–testis barrier closure in SCCx43KO$^{-/-}$ mice, resulting in the absence of migration of germ cells, could be sustained by the active proliferation of early spermatogonia in the testes of these transgenic mice (Sridharan et al., 2007a; 2007b). The current ultrastructural analysis showing an increased number of tight and adherens junctions at the level of the blood–testis barrier in the SCCx43KO$^{-/-}$ testis supports this idea but further experiments are needed to verify such a hypothesis.

Another point that should be discussed is to determine whether Cx43 control tight and adhesion junction proteins in a GIJC-dependent or -independent manner (see schema depicted in Fig. 8). Thus, the effect of two blockers of gap junction coupling was analyzed in the SerW3 Sertoli cell line. The present data clearly showed that both agents markedly altered the levels of occludin, N-cadherin and β-catenin, suggesting that the effect of Cx43 could be channel-dependent. Our data does not, however, exclude the possibility that Cx43, in addition to control tight and adherens junction proteins through gap junctions, could also partly regulate these proteins in a gap junction independent manner through up-regulation of MAGI-1 and JAM-1 which may play a crucial role in formation and assembly of tight junctions as recently suggested (Kojima et al., 2007). Another possibility could be that, during uncontrolled proliferation in the SCCx43KO$^{-/-}$ testis, Sertoli cells produce increasing levels of cytokines that are known to play a critical role in the disruption of the integrity of the Sertoli cell tight junction permeability (reviewed by Li et al., 2009b). Lastly, at this time we cannot rule out that Cx43-hemichannels, which have the capacity to transfer small signaling molecules, could also participate in this control. Indeed, there is major information in the literature suggesting a possible role for undocked hemichannels in several cellular processes such as the release of ATP, NAD^+, prostaglandin and glutamate, the intercellular calcium wave propagation, cell-volume regulation and transduction of survival signals (reviewed in Spray et al., 2006). In addition, we have no information on the nature of the intercellular signals that pass through gap junction channels or hemichannels and might be required for maintaining the other protein component of the blood–testis barrier. However, the present findings that ZO-1 levels were differently altered in response to two gap junction blockers, such as oleamide and α-GA that blocks gap junction functionality with or without altering Ca^{++} waves (Guan et al., 1997), allow us to suspect Ca^{++} as a possible candidate. This hypothesis is in agreement with previous data demonstrating that Ca^{++} is critical for adherens and tight junction function at the blood–brain barrier (Brown and Davis, 2002).

The integrity of epithelial and endothelial barriers is critical to human health, but there is still a lack of detailed mechanistic knowledge of how a barrier is locally controlled or is able to respond to pathological and pharmacological effectors. Altogether our data, based on in vivo and in vitro approaches, demonstrate that Cx43 is capable of regulating other proteins of the intercellular junctional complexes that occur between adjacent cells. In the testis, this control is not crucial for maintaining the blood–testis barrier integrity, since this structure is effective in the SCCx43KO$^{-/-}$ testis, but it probably interferes with the barrier dynamics. Indeed, since the junctions are extended and the germ cell differentiation altered, it seems likely that the resulting effects are due to junctional protein dysregulations consequent to their overexpression. This could lead to impairment of the transit of germ cells across the seminiferous epithelium and consequently to altered spermatogenesis in Sertoli cell Cx43 knockout mice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.07.014.

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